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Running Title: Single dose gene therapy for CF

The vast majority of treatments for cystic fibrosis (CF) target the downstream consequences of the disease and are incompletely effective. The success of the cystic fibrosis transmembrane conductance regulator (CFTR) potentiator, ivacaftor, has illustrated the clinical benefits arising from restoration of CFTR protein function (1). This agent is applicable as a monotherapy for a minority of patients with specific, rare mutations. *CFTR* gene therapy, a mutation-independent alternative, has demonstrated proof-of-principle for gene transfer in animal models and human trials, but only one study (using a viral vector) has unsuccessfully assessed whether clinical outcomes can be improved (2). In preparation for a Phase IIb clinical trial of repeatedly administered, non-viral, liposome-mediated *CFTR* gene transfer assessing clinically-meaningful outcomes (3), the UK CF Gene Therapy Consortium (www.cfgenetherapy.org.uk) undertook a single application, safety and dose ranging study (NCT00789867

Some of the results of these studies have been previously reported in the form of abstracts. (4, 5)

The chosen plasmid DNA expresses CFTR under the control of the hCEFI sequence (6), a modified EF1a promoter aiming for extended duration of expression (7) and was rendered CpG-free to minimise a host inflammatory response (6). The cationic lipid, GL67A, was chosen on the basis of extensive preclinical testing (8). Following informed consent, adult CF subjects received a single nebulised +/- nasal dose of pGM169/GL67A. Reconstitution and preparation of pGM169/GL67A was undertaken on the day and doses delivered in sealed negative-pressure cubicles following pre-treatment with inhaled salbutamol (albuterol). Pre-planned adjunctive therapies including ibuprofen, prednisolone or paracetamol were administered to some patients.

The primary outcome of the clinical study was safety; assessment included examination, standard haematology/biochemistry, adverse events, spirometry, lung clearance index, chest CT, gas transfer, bronchial biopsy histology and immune markers. pGM169-specific DNA and mRNA were measured on nasal and lower airway brushings, with potential difference also measured bronchoscopically and nasally. For the latter, 'responders' were defined as demonstrating chloride secretion ≥ 5 mV more than their mean pre-dose value and greater than any of their pre-dose responses.

A total of 35 subjects (Table 1) received a nebulised dose (5 ml n=8, 10 ml n=10, 20 ml n=17) via an AeroEclipse II (Trudell) breath-actuated nebuliser (9). Three subjects undertook slow delivery (~ 75 min versus 25 min for each 5 ml). Standard spray devices were used for nasal delivery (2 ml, n=21). Based on pre/post device weighing, 88.7 (2.9)% of expected nebulised dose and 94.5 (15.0)% of expected nasal dose was delivered. There were two serious adverse events (SAEs): one occurred following the pre-dosing bronchoscopy (swelling of the uvula related to intubation) and led to observation overnight in hospital; the other was an episode of pancreatitis occurring around day 10 post dosing (10 ml nebulised cohort). The subject was exocrine pancreatic sufficient and had likely experienced previous similar, but undiagnosed episodes.

Overall, in the trial, 94.3% of subjects experienced at least one adverse event (AE), the majority of which were mild to moderate in severity and resolved spontaneously, or with standard antipyretics. The commonest occurred on the day of dosing and largely resolved within 24-48 hours (Table 1): Typically, within the first few hours post-dosing, a mild, self-limiting flu-like systemic response was seen, most frequently in the 20 ml patients. This was not affected by slow delivery or co-administration of ibuprofen or prednisolone, but was clearly dose-related and reduced by paracetamol. Symptoms of headache and/ or tiredness were reported by 82%, 70% and 13%, and raised serum inflammatory markers recorded in 100%, 60% and 63% of the 20, 10 and 5 ml groups respectively, with dose related trends in maximal values . No patient dosed with 5 ml had a temperature >38^{o}C (Table 1)A relatively asymptomatic, dose-related, restrictive drop in spirometry was also observed, with no change in respiratory rate or oxygen saturation. No patient dosed with 5 ml showed a >20% relative fall in FEV_1 (Table 1). The 20 ml group showed a small, significant ($p<0.05$) mean (SD) drop in gas transfer (transfer factor for carbon monoxide corrected for alveolar volume and haemoglobin concentration (KCOc)) on day 2 of 4.5(6.0)% which had returned to baseline values by day 14. No changes were seen in the other cohorts. Two of the 20 ml patients had small areas of ground glass opacity reported on their day 2 chest CT scans, which had resolved by day 14. No significant changes were seen in endobronchial histology (20 ml; n=10).

Consistent with the proposed excretion route for lipids, small but significant serum creatinine rises *within* the normal range could be detected 8 hours post dosing in the 20 and

10 ml group but not the 5 ml cohort; there were no other biochemical changes. Bilirubin rose on day 1 in all dosing groups, as with creatinine remaining within the normal range, and normalised by day 2. There was no evidence of immune responses based on double-stranded DNA antibodies or human CFTR-specific T cells.

Lung clearance index (LCI), a sensitive marker of pulmonary dysfunction (10) was included as a safety assay. Fourteen 20 ml patients with paired pre and 28 days post-dosing values, showed a small, but significant increase (ie a deterioration; Fig1a). In contrast, and unexpectedly, on post hoc analysis, 11/14 patients in the lower dosing groups (5 and 10 ml) showed a small but significant improvement (Fig 1a).

With respect to bronchial samples, ten patients (all 20 ml) had paired pre- and post-dosing bronchoscopies. pGM169-specific DNA was detected in all bronchial brushing samples at levels ~x1000-fold higher than in the nasal samples. pGM169-specific mRNA was detected in 2/10 post-dosing samples. Paired bronchoscopic potential difference (PD) measurements were interpretable on 8/10 patients. There was a trend towards an increase in chloride secretion (Fig 1b), but no changes in sodium-related parameters.

With respect to nasal samples, pGM169-specific DNA was detected in all 15 brushing samples taken between day 2 and day 14 post-dosing and in 2/6 samples at day 28. pGM169-specific mRNA was detected in 3/21 post-dosing samples, all positive samples being observed at either day 14 (n=2) or 28 (n=1). In keeping with previous published data, there were no changes in sodium parameters on nasal PD. In contrast, 6/16 subjects (37.5%) demonstrated a 'response' in terms of chloride secretory capacity. Responses were seen most commonly in the zero chloride perfusion phase and at the 14 day time point; they were of sustained duration in one subject. (Fig 1c).

These data were important in informing the design of the Phase IIb trial. Thus, based on these findings, 5 ml was selected as the optimal dose with paracetamol being used as an adjuvant to minimise the risk of unblinding. Although well-tolerated, the side effects of the 20 ml doses were considered prohibitive for use in a repeated administration trial. We consider that the efficient delivery of large volumes of viscous fluid into the airways led acutely to both the flu-like and restrictive responses, analogous to those seen post-bronchoalveolar lavage, and masking the effect of plasmid DNA CpG depletion. At lower

volumes, the latter effect was ‘revealed’, allowing safe dosing of 5 ml. The unexpected improvement in LCI after only one administration at the lower doses was intriguing; larger numbers and longer follow-up are needed to confirm or refute this finding. The variable responses both in molecular and CFTR functional terms underscore the technical challenges inherent in these assays and the limited sensitivity to low levels of gene expression (11). The clean safety profile and encouraging improvements in a sensitive measure of airway health, lend support to progression to a Phase IIb, multidose trial designed to detect clinical improvements following prolonged administration.

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